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TO ALL WHOM IT MAY CONCERN:

Be it known that we, Robert Shipman, a citizen of the Canada residing in Mississauga, Canada, James Leushner, a citizen of Canada residing in North York, Canada, and James M. Dunn, a citizen of Canada residing in Scarborough, Canada, whose post office addresses are #2-6240 Montevideo Rd., Mississauga, ON L5N 2N7, Canada, 84 Sylvan Valley Way, North York, ON M5M 4M3 Canada, and 117 Citadel Drive, Scarborough M1K 4S8 Canada, respectively, have invented an improvement in

METHOD AND REAGENTS FOR TESTING FOR  
MUTATIONS IN THE BRCA1 GENE

of which the following is a

SPECIFICATION

This application is a continuation in part of US Patent Application Serial No. 08/271,946 filed July 8, 1994 which is incorporated herein by reference.

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BACKGROUND OF THE INVENTION

Genetic testing to determine the presence of or a susceptibility to a disease condition offers incredible opportunities for improved medical care, and the potential for such testing increases almost daily as ever increasing numbers of disease-associated genes and/or mutations are identified.

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One such disease-associated gene is the BRCA1 gene. Mutations in the BRCA1 gene have been shown to be linked to breast and ovarian cancer. Miki et al., "A Strong Candidate for the Breast Cancer and Ovarian Cancer Susceptibility Gene BRCA1", *Science* 226: 66-71 (1994).

Since the identification of the BRCA1 gene, researchers have tested many individuals using sequencing, single-stranded conformational polymorphism, allele-specific oligonucleotide hybridization and heteroduplex analysis to identify BRCA1 mutations in families with a history of breast and ovarian cancer. Shattuck-Eidens et al., "A Collaborative Survey of 80 Mutations in the BRCA1 Breast and Ovarian Cancer Susceptibility Gene", *J. Amer. Med. Assoc.* 273: 535-541 (1995). Diagnostic screening to identify persons with these mutations, and thus with an apparent genetic predisposition to breast and ovarian cancers offers the opportunity for increased monitoring of high-risk patients which should lead to earlier detection of cancer. Such early detection improves the likelihood of successful treatment and the likelihood of long-term post-detection survival. On the other hand, large scale screening could be prohibitively expensive, absent a easily-performed, low-cost test for mutations in the BRCA1 gene.

It as an object of the present invention to provide a screening methodology which can be used to provide low-cost testing for mutations in the BRCA1 gene.

It is a further object of the present invention to provide reagents, particularly primers and primer cocktails, which can be used in testing for mutations in the BRCA1 gene.

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#### SUMMARY OF THE INVENTION

In accordance with the present invention, samples are tested for mutations in the BRCA1 gene using a hierarchical approach. First, each sample is amplified in one or more multiplex PCR amplification reactions. Each multiplex PCR reaction produces a mixture of amplified fragments. The sizes and amounts of these fragments are evaluated and compared to standard values reflecting the sizes and amounts of fragments produced when the same multiplex amplification is performed on the wild-type BRCA1 gene. Differences between the observed fragment sizes and/or amounts and those for the wild-type gene are indicative of a mutation with the BRCA1 gene of the sample.

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The next step in the method of the invention is sequencing of one or more regions within the BRCA1 gene. In accordance with the hierarchical method, such sequencing will be performed on samples where no mutation was detected by analysis of the multiplex PCR fragments.

#### BRIEF DESCRIPTION OF THE DRAWINGS

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Fig. 1 illustrates an embodiment of the sequencing step of the method of the invention; and

Figs. 2A and 2B illustrate two embodiments of the method of the invention in which multiplex amplification and sequencing are performed sequentially on the same initial aliquot of sample.

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#### DETAILED DESCRIPTION OF THE INVENTION

To date, over thirty BRCA1 mutations have been identified in the breast cancer families which have been studied. A substantial number of these mutations are insertion or deletion mutations. Furthermore, 75% of the currently known mutations are found in exon 11 and an additional 5% are found in exons 2 and 20. This type of distribution makes BRCA1 well-suited for analysis using the type of hierarchical analysis described in co-pending US Patent Application Serial No. 08/271,946. Use of a hierarchical analysis provides highly accurate test results at a reduced cost per patient.

The first step in the hierarchical analysis is multiplex amplification and fragment length analysis of at least exons 2, 11 and 20 of the BRCA 1 gene. For multiplex amplification, a sample to be evaluated is combined with a several amplification primers. Amplification primers are selected to hybridize with the known sequence of the introns or exons of the BRCA1 gene. This sequence can be found online at the Breast Cancer Information Core, which has the following URL:  
[http://www.nchgr.nih.gov/Intramural\\_research/Lab\\_transfer/Bic/](http://www.nchgr.nih.gov/Intramural_research/Lab_transfer/Bic/).

Information about the cDNA sequence can also be found from GenBank Accession No. U14680 or Genome Data Base Accession No. GDB: 126611. While considerable variation is theoretically possible in the sequence of these primers, the practical requirements for multiplex amplification and fragment analysis mean that primers cannot be simply selected at random. These requirements impose at least the following limitations on primers used in the method of the invention:

(1) in order to avoid the possibility of false positive results the primer pair, i.e., the combination of the 5'-primer and the 3'-primer for any given exon must be unique to the BRCA1 gene so that only the BRCA1 gene will be amplified. This means that the primer sequences will be generally somewhat longer than the minimum which can be used as an amplification primer. Preferred primers are from 18 to 23 nucleotides in length, without internal homology or primer-primer homology;

(2) It is also desirable for the primers to form more stable duplexes with the target DNA at the primers' 3'-ends than at their 5'-ends, because this leads to less false priming. Stability can be approximated by GC content, since GC base pairs are more stable than AT pairs, or by nearest neighbor thermodynamic parameters. Breslauer et al., "Predicting DNA duplex stability from base sequence", *Proc. Nat'l Acad. Sci. USA* 83: 3746-3750 (1986).

(3) To ensure complete amplification of each exon, the two primers of a pair are preferably selected to hybridize in the introns immediately flanking the exon to be amplified using the primer pair.

5 (4) Primer pairs are advantageously selected to have similar melting temperatures. Further, multiplex pools should contain primer pairs with approximately the same thermal profile, so that they can be effectively coamplified together. This goal can be achieved by having groups of primer pairs with approximately the same length and the same G/C content.

10 (5) The length of the gene region between the primer binding sites on a normal BRCA1 gene should be different for each exon to be multiplexed as a group. Differences of only one base in length are sufficient, provided a high resolution gel capable of resolving one base differences is used in analyzing the amplification products. However, greater differences in length are preferred, to avoid confusion in interpreting fragment sizes resulting from an insertion or deletion mutation.

15 20 Table 1 shows a series of amplification primers for various exons of the BRCA1 gene which meet these criteria when grouped into one of the multiplex groups as shown in the Table. It will be understood in the art that the same amplification products could be produced using primer pairs complementary to those set forth in Table 1. Because of the large size of exon

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11, this exon is preferably amplified in multiple overlapping  
fragments using primers which bind to the exon in an  
interlocking manner so that all regions of the exon are  
amplified and analyzed, rather than using intron primers. This  
5 provides a more robust analysis, since amplification of shorter  
regions provides more reliable results, and also facilitates  
localization and identification of detected insertion or  
deletion mutations. Melting temperatures and optimum annealing  
temperatures in Table 1 are calculated assuming a salt  
10 concentration of 50 mM and 250 pM primer concentration.  
Conditions of actual use may be different, for example primer  
concentration of 200 nM to 1  $\mu$ M will normally be used. Because  
of variations in primer and/or salt concentrations,  
experimental optimization using the calculated temperature as a  
15 starting point may be desirable.

While the foregoing set of primers provides the  
desirable ability to coamplify regions of the BRCA1 gene for  
fragment length analysis, it will be appreciated that other  
primers sets can be used as well based upon the known sequence  
20 of the BRCA1 gene by evaluating the uniqueness of the primer  
sequences and determining the predicted melting temperature for  
each primer. This can be accomplished in several ways. For  
example, the melting temperature,  $T_m$  can be calculated using  
either of the following equations:

$$T_m(^{\circ}\text{C}) = 81.5 + 16.6 \times \log [\text{Na}] + 0.41 \times (\% \text{GC}) - 675/\text{length}$$

where [Na] is the concentration of sodium ions, and the %GC is in number percent, or

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$$T_m (^{\circ}\text{C}) = 2 \times (\text{A} + \text{T}) + 4 \times (\text{G} + \text{C})$$

where A, T, G, and C represent the number of adenosine, thymidine, guanosine and cytosine residues in the primer.

10 Alternatively, OLIGO™ software can be used to calculate  $T_m$ , nearest neighbor and  $\Delta H/\Delta G$  values. In general, primers for coamplification should be selected to have predicted melting temperatures differing by less than 4°C.

15 To perform the multiplex fragment analysis, each selected set of primers is combined with an aliquot of sample in a reaction mixture containing a template-dependant DNA polymerase, such as Taq polymerase, T7 DNA Polymerase, or Thermo Sequenase®; deoxynucleoside triphosphate feedstocks (A, C, G and T); and an appropriate buffer to permit extension of  
20 the primers. The resulting reaction mixture is thermally cycled through multiple cycles of annealing (performed at or below an average of the optimum annealing temperatures listed in Table 1), primer extension (performed at about 70 to 72° C) and denaturation (performed at a temperature of around 95°C).  
25 In practice, each reaction condition is optimized using the



predicted temperatures as a starting points, and varying the temperature within a range from about 44 to 66 °C.

After sufficient cycles to produce detectable levels of amplification products, the product mixture is loaded onto a separation matrix, e.g., a polyacrylamide gel, more specifically a PAGE sequencing gel, and separated on the basis of fragment size. Labeled amplification primers, particularly of amplification primers labeled with fluorophores such a fluorescein, facilitate detection of the separated bands within the separation matrix. The size of each detected fragment is determined, and compared to the expected size of the fragments for the multiplex amplification. Deviations in size or loss of a band reflect the presence and size of an insertion or deletion mutation involving the amplified fragment forming that band. Reduction in the intensity of a band generally reflects loss of one copy of the amplified exon or region in the sample.

The amplification reaction are preferably performed in a quantitative manner. This means that, for maximum effectiveness in the method of the present invention, the amplification of the exons in the sample should be carried out only for a number of cycles during which doubling of DNA is still being achieved.

Because of the frequency of insertion and deletion mutations among the known mutations in the BRCA1 gene, the fragment analysis test may, in many instances provide all the

information that is needed to provide a diagnostic result.  
Where no insertion or deletion mutation is detected in the  
fragment analysis, however, the sample is further evaluated to  
determine the sequence of the one or more exons (or partial  
exons) of the BRCA1 gene.

Sequencing can be performed in known manner, although  
it will preferably be performed using a chain-termination  
sequencing reaction as originally described by Sanger et al.  
More preferably, the sequencing will be performed by  
amplification and sequencing of an exon (or portion thereof) of  
the BRCA1 gene using the methodologies disclosed in US Patent  
Application No. \_\_\_\_\_ filed May 1, 1996 (Attorney  
Docket No. VGEN.P-020), which is incorporated herein by  
reference. This amplification and sequencing procedure can be  
carried out on a fresh aliquot of the original sample, or on  
aliquots of one or more of the multiplex fragment analysis  
product mixtures.

Fig. 1 illustrates a first embodiment of the sequencing  
step of the present invention. As shown in Fig. 1, a sample  
containing a target nucleic acid polymer which is to be  
amplified and sequenced is combined with an amplification  
mixture containing two primers for an exon or portion of an  
exon of the BRCA1 gene, a mixture of dNTP's and thermostable  
polymerase in a buffer suitable for amplification. The primers  
may be the same primers as disclosed in Table 1, or other

primers which hybridize selectively with the selected part of the BRCA1 gene.

5 The mixture is amplified through an initial set of cycles, for example 15-40 cycles. At this stage reagents for forming chain termination products, namely a dideoxynucleoside triphosphate (ddNTP) and optionally additional thermostable polymerase, dNTP's and a labeled sequencing primer are added and additional cycles (for example another 15-20 cycles) are performed during which both amplification and the formation of chain termination products occurs. The sequencing primer may be one of the primers disclosed in Table 1 for the exon or exon portion or some other primer which hybridizes specifically with the amplified portion of the BRCA1 gene, e.g., a nested primer. At the end of these cycles, the product mixture is evaluated to determine the lengths of the chain termination products, and hence the positions of the particular base corresponding to the ddNTP within the target nucleic acid polymer.

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25 The thermostable polymerase used in at least the sequencing step of this amplification and sequencing process, and preferably in both steps, is one which incorporates dideoxynucleosides into an extending oligonucleotide at a rate which is no less than about 0.4 times the rate of incorporation of deoxynucleosides in the same amplification mixture. The commercially available enzyme Thermo Sequenase® is such an enzyme.

A variation of this method which may be advantageous is the use of asymmetric amplification to preferentially amplify one strand of the target nucleic acid. In this case, the primer which will produce the desired sequencing template strand is combined with the sample in an amount greater than the other primer, e.g., a 10 to 50-fold excess. More amplification cycles may be required to take full advantage of asymmetric amplification.

It may also be advantageous to biotinylate one of the primers used for amplification. When amplification is carried out with biotinylated primers, a partial separation of reagents can be accomplished prior to the introduction of the sequencing reagents by capturing the biotinylated amplification products on an avidin or streptavidin-coated support, separating the liquid medium from the support and replacing the liquid medium with the sequencing reagents. In a preferred use of this approach, the biotinylated products are captured on metal or magnetic beads, which are captured with a magnet to facilitate separation of the amplification liquid. While this step is not necessary to the method, and is not intended to accomplish complete removal of the amplification reagents, the use of this step can improve the sensitivity of the procedure by reducing the number of background oligonucleotides, particularly where a separate labeled-sequencing primer is added with the ddNTP.

5 Figs. 2A and 2B illustrate two embodiments of the method of the invention. As shown in Figs. 2A and 2B, a patient sample 1 is first subjected to multiplex PCR to produce a complex mixture of amplification products using sets of amplification primers for the BRCA1 gene as disclosed in Table 1. The products of this mixture are analyzed, to detect insertion or deletion mutations. If the results of the fragment length analysis fail to show a mutation, the sample 1 is further analyzed by sequencing a selected exon or exons.

10 An aliquot of the original multiplex PCR amplification mixture is used as the starting material for multiple cycles of combined amplification and sequencing. Thus, the multiplex PCR amplification mixture is combined with amplification and/or labeled sequencing primers and amplified and sequenced in a single reaction vessel. Preferably, the multiplex amplification PCR is performed using capturable primers (for example biotin-labeled primers) and separated from the multiplex amplification reagents using affinity beads (e.g. avidin-coated beads) prior to the addition of the amplification/ sequencing reagents. (Fig. 2B). Additional aliquots of the multiplex reaction mixture may be processed to sequence different regions if no mutation is detected in the first sequencing step.

20 It should be noted that the multiplex reaction performed in the first step of this embodiment makes use of labeled primers. Fluorescence from these primers may interfere

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with observation of a few peaks in the sequencing ladder. This interference can be minimized by utilizing a nested sequencing primer, which produces fragments having a maximum length which is shorter than the multiplex amplification products, or by the utilization of distinguishable labels for the multiplex amplification and sequencing primers.

The fragment analysis and sequencing steps of the present invention may also be advantageously combined with additional analytical steps for evaluation mutations in the BRCA1 gene. For example, a ligation analysis of the type described in the US Patent Application No. 08/590,503 which is incorporated herein by reference can be used before or after the fragment length analysis to detect mutations in some or all of the exons of the BRCA1 gene. Briefly, this technique makes use of a set of oligonucleotide probes which hybridize in series along the length of the exon or region being evaluated. The ligation of the probes together forms a ligation product, the size of which is evaluated. When the gene or gene fragment being analyzed corresponds to the normal sequence and thus perfectly matches the probes, all of the probes in the set are ligated together, and the ligation product has a certain resulting size. When a mutation appears in the gene, the hybridization of the probe overlapping the mutation is impaired, with the result that some or all of the ligation product is of smaller size. By evaluating the size of the

ligation product, both the existence of a mutation and its approximate position can be identified.

Use of CLEAVASE (Third Wave Technologies, Inc. Madison WI) provides another diagnostic technique which can be used according to the invention to identify mutations in BRCA1 by determining the sizes and amounts of amplified exon fragments. CLEAVASE is an endonuclease which cuts single stranded DNA (ssDNA) molecules. Since mutant ssDNA adopts a different conformation from wild-type ssDNA, the CLEAVASE digestion products show a different array of fragments. This array of fragments can be separated and examined by electrophoresis, much like multiplex PCR fragments. The advantage of CLEAVASE is that it can detect single base substitution mutations as well as insertions and deletions. Therefore, it detects fewer false negatives than multiplex PCR, though it does not locate mutations as precisely as sequencing.

VGEN. P-028-US

TABLE 1

Exon	Prod Size (bp)	Optimum Annealing Temp (°C)	Seq. ID No.	Primer	Sense/Anti-sense	Tm (°C)	MP P-1
1	234	59.1	1	GGTAGCCCCCTTGGTTCCGTG	sense	59.3	A
			2	ACGCCAGTACCCAGAGCATC	anti	57.7	
2	236	46.8	3	AATGATGAAAAATGAAGTTGTC	sense	43.4	B
			4	GTTTCATTTGCATAGGAGATAA	anti	44.1	
2	275	47.7	75	AAACCTTCCAAATCTTCAAAT	sense	46.9	C
			76	TTCGTGTTTCATTTGCATAGGAG	anti	47.3	
2	286	46.9	75	AAACCTTCCAAATCTTCAAAT	sense	46.9	B
			77	TGTAAGGTCAATTTCTGTTCAT	anti	43.8	
3	116	47.3	5	GAGCCTCATTTATTTTCTTTT	sense	44.9	C
			6	TGAAGGACAAAAACAAAAGCT	anti	48.8	
4	204	49.2	7	ACCTTAAATTTTTCACAGCT	sense	45.2	E
			8	CTCTACAGAAAAACACAAAATT	anti	41.0	
5	202	46.6	9	GCCTTTTGAGTATCTTTCTA	sense	43.2	B
			10	TTCTACTTTTCTCTACTGTGGT	anti	42.8	
6	233	48.9	11	AGGTTTCTCTACTGTTGCTGCA	sense	49.5	D
			12	CAGCACTTCACTCTCATTCTT	anti	46.1	



TABLE 1

Exon	Prod Size (bp)	Optimum Annealing Temp (°C)	Seq. ID No.	Primer	Sense/Anti-sense	Tm (°C)	MP P-1
7	218	47.3	13	CATACATTTTCTCTAACTGC	sense	41.5	C
			14	GAAGAAAGAAGAAAACAAATGG	anti	45.5	
8	193	50.4	15	AGGAGGAAAAGCACAGAACTG	sense	50.4	F
			16	TACTTAAAAAACCTGAGACCC	anti	45.2	
9	197	46.0	17	CAAGTACATTTTTTTAAACCCCT	sense	43.2	B
			18	AAAGAGAGAAACATCAATCCT	anti	44.1	
10	227	48.9	19	TTTGACAGTCTGCATACATG	sense	46.0	D
			20	CAAATGGTCTTCAGAATAATC	anti	43.4	
11a	314	49.6	21	CTCCAAGGTGTATGAAGTATG	sense	44.0	I
			22	CAGCCTTTTCTACATTTCATTC	anti	46.2	
11b	348	49.8	23	ATTACAGCATGAGAACACAGCAG	sense	47.2	J
			24	GAGTCATCAGAACCTAACAGT	anti	42.2	
11c	340	49.1	25	ATAGCAGCATTCAGAAAGTTA	sense	44.2	H
			26	TCAGTAACAAATGCTCCTATA	anti	42.3	
11d	314	50.2	27	CTCCCCAACTTAAGCCATGTA	sense	51.2	K
			28	TCGAGTGATTCATTGGGTTA	anti	46.8	

VGEN.P-028-US

TABLE 1

Exon	Prod Size (bp)	Optimum Annealing Temp (°C)	Seq. ID No.	Primer	Sense/ Anti-sense	Tm (°C)	MP P-1
11e	325	49.2	29	TGGTCATGAGAATAAAACAAA	sense	45.1	I
			30	TGGCAATTTGGTTGTACTTTT	anti	49.4	
11f	352	51.1	31	AAGCCCACCTAATTGTACTGA	sense	48.4	L
			32	TTTGGGCTCTTCAGCATTTAT	anti	50.8	
11g	307	48.3	33	AAGAAGAGAAACTAGAAACAG	sense	39.5	H
			34	AATGGATACTTAAAGCCTTCT	anti	44.3	
11h	332	49.2	35	AAGGGACTAATTCATGGTTGT	sense	47.2	I
			36	GTCGTACAGGCTTGATATTA	anti	41.5	
11i	255	50.4	37	TGAATGTGAACAAAGGAAGA	sense	47.4	K
			38	ATGGGAAAAAGTGGTGGTATA	anti	48.2	
11j	346	48.6	39	AACGAAACTGGACTCATTTACT	sense	45.0	H
			40	TGTTTCTACCTAGTTCTGCTT	anti	43.2	
11k	340	49.4	41	TGGGCTCCAGTATTAATGAAA	sense	49.4	I
			42	TCAGCAAACTAGTATCTTCC	anti	43.5	
11l	313	49.8	43	CATGCATCTCAGGTTTGTTCT	sense	49.3	J
			44	TATGCCTAGTAGACTGAGAAG	anti	40.9	

TABLE 1							
Exon	Prod Size (bp)	Optimum Annealing Temp (°C)	Seq. ID No.	Primer	Sense/Anti-sense	T <sub>m</sub> (°C)	MP P-1
11m	312	52.7	45	GCTTCCCTGCTTCCAACACTT	sense	55.0	L
			46	TGCCCTCATTTGTTTGAAGAA	anti	52.5	
11n	277	52.1	47	ACAGTGCAGTGAATTGGAAGA	sense	49.7	K
			48	CTCCCCAAAAGCATAAACATT	anti	50.5	
12	191	49.2	49	GC GTTATAGTCTGCTTTTAC	sense	43.9	E
			50	TTGGAGTGGTATTCTTTTAAG	anti	43.7	
13	267	50.5	51	TATTTTCAATTTTCTTGGTACCA	sense	44.6	F
			52	ATAAAGGGAAGGAAAGAAATT	anti	47.9	
14	251	46.7	53	GAATTATCACTATCAGAACAA	sense	38.6	B
			54	CAATCAGAGTTCAATATAAAT	anti	38.3	
15	393	47.4	55	CCAGCAAGTATGAAATGTCCT	sense	48.7	C
			56	CTTTATGTAGGATTCAGAGTA	anti	38.3	
16	559	49.6	57	CTTAACAGAGACCAGAACTTT	sense	42.4	E
			58	TTTCCAGAATGTTGTTAAGTC	anti	43.9	
17	212	48.5	59	CTAGTATTTCTGAGCTGTGTGC	sense	44.3	D
			60	CCTCGCCCTCATGTGGTTTAT	anti	53.3	

TABLE 1							
Exon	Prod Size (bp)	Optimum Annealing Temp (°C)	Seq. ID No.	Primer	Sense/ Anti-sense	Tm (°C)	MP P-1
18	258	48.9	61	CTCTTTAGCTTCTTAGGACAG	sense	42.8	D
			62	CTCAAGACTCAAGCATCAGCA	anti	50.6	
19	215	50.5	63	TGTGAATCGCTGACCTCTCTA	sense	50.0	F
			64	AAGTGGTGCATTGATGGAAGG	anti	53.9	
20	169	52.0	65	TCTCTTATCCTGATGGGTGTG	sense	49.0	G
			66	ATACAGAGTGGTGGGTGAGA	anti	50.8	
21	167	56.4	67	CAGTGGTGGATCTCAGCTCA	sense	56.2	A
			68	AAGGCTGGTGGTGGAACTCTG	anti	55.8	
22	273	51.0	69	TAGAGGCCCTGGTTAAGTAT	sense	49.3	G
			70	GAGAAGACTTCTGACGCTACC	anti	46.6	
23	152	51.1	71	CCTACTTTGACACTTTGAATG	sense	44.3	G
			72	AATGTGCCAAGAAGTGTGCTA	anti	50.1	
24	252	50.9	73	TAATCTCTGCTTGTGTTCTCT	sense	43.4	F
			74	GTAGCCAGGACAGTAGAAGGA	anti	47.9	

EXAMPLE 1

Individual exons of the BRCA1 gene are amplified as follows.

5           5 ul of patient sample genomic DNA (20 ng/ul) is  
combined with 2 ul 10X PCR Buffer, 0.6 ul 50mM Mg2+, 0.4 ul 10  
mM dNTP mix (containing each of the 4 dNTPs), 1 ul DMSO (100%),  
and 8 ul ddH2O. 2 ul of a 50 ng/ul mixture of an amplification  
primer pair (i.e. 100 ng each primer), one of which is labeled  
10 with a detectable label, are added to the mix. The  
amplification primer pair is one of the pairs of exons  
designated in table 1 as being specific for a particular exon.  
A suitable label is fluorescein, which can be detected on an  
A.L.F. automated DNA sequencer (Pharmacia, Inc., Piscataway,  
15 N.J.).

The mixture is prepared on ice. Addition of 1ul Taq  
Polymerase (1U/ul) (Roche Molecular Systems, Inc.), bringing  
the total volume to 20 ul, is followed by thermal cycling as  
follows:

20 Initial denaturation   94C   10 min

Cycle (20 - 35 times):

anneal	*47C	40 sec
extend	72C	60 sec
denature	94C	30 sec

Final extension            72C 10 min then hold at room  
temperature.

\* annealing temperature may vary within about 6° C depending on  
empirically determined annealing temperature for the specific  
primer pair(s).

An equal volume of Stop Solution comprising Formamide  
and a visible dye is added after the reaction is complete. 6  
ul of this mixture is loaded into a single lane of an A.L.F.  
Sequencing Gel, and the reaction products are detected.

### Example 2

Multiplex amplification of selected exons or parts of  
exons of BRCA1 is achieved as follows:

5 ul of patient sample genomic DNA (20 ng/ul) is  
combined with 2 ul 10X PCR Buffer, 0.6 ul 50mM Mg2+, 0.4 ul 10  
mM dNTP mix (containing equimolar amounts of each of the 4  
dNTPs), 1 ul DMSO (100%), and 8 ul ddH2O. 2 ul of a mixture  
containing 50 ng/ul of a multiplex set of primers as indicated  
in Table 1, (i.e. 100 ng each primer), one of each pair being  
labeled with a detectable label, are added to the mix. A  
suitable label is fluorescein, which can be detected on an  
A.L.F. automated DNA sequencer (Pharmacia, Inc., Piscataway,  
N.J.).

The mixture is prepared on ice. Addition of 1ul Taq Polymerase (1U/ul) (Roche Molecular Systems, Inc.), bringing the total volume to 20 ul, is followed by thermal cycling as follows:

5 Initial denaturation 94C 10 min

Cycle (20 - 35 times):

anneal \*47C 40 sec

extend 72C 60 sec

denature 94C 30 sec

10 Final extension 72C 10 min then hold at room temperature.

\* annealing temperature may vary within about 6°C depending on empirically determined annealing temperature for the specific primer pair(s).

15 An equal volume of Stop Solution comprising formamide and a visible dye is added after the reaction is complete. 6 ul of this mixture is loaded into a single lane of an A.L.F. Sequencing Gel, and the reaction products are detected.

### 20 Example 3

Sequencing of an individual exon of BRCA1 is achieved as follows.

Amplified, biotinylated PCR product is prepared as a template for sequencing by generating the following mixture:

25 300 ng genomic DNA (patient sample); 1X Taq polymerase Buffer

(final: 10mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5mM MgCl<sub>2</sub>, 0.001% gelatin); and 0.2mM each dNTP. To this mixture is added 8 pMol of each primer selected from table 1 to amplify a specific exon of BRCA1, one of which primers is biotinylated. The reaction mixture is kept on ice until the addition of 2.5U Taq DNA polymerase. The final volume is 25 ul.

This reaction mixture is thermal cycled in a Perkin Elmer 9600 as follows:

94°C 2 min x1 cycle

94°C 30 sec

\*50°C 30 sec x35 cycles

65°C 2 min

65°C 7 min x1 cycle

\* annealing temperature may vary within about 6°C depending on empirically determined annealing temperature for the specific primer pair(s).

At the end of the reaction, a 5 ul aliquot may be taken and observed on a 1% agarose gel containing ethidium bromide to assess integrity of the amplification reaction.

If the PCR product appears satisfactory, the reaction buffer is exchanged using streptavidin/magnetic beads as follows:



1. take 8 ul of streptavidin beads (Dyna1), wash with 50 ul 2x BW buffer
2. resuspend beads in 10 ul of 2x BW buffer
3. remove 10 ul of PCR product from above and mix with washed beads.
4. sit at RT for up to 1 hour with periodic mixing by gently tapping side of tube
5. place on magnetic rack, allow PCR bound-beads to separate and remove supernatant. Wash with 50 ul of 1x BW buffer, separate on magnetic rack and remove supernatant. Repeat with 50 ul of TE.
6. resuspend bound beads in 10 ul of dH2O.
7. use 3 ul for cycle sequencing

\*2xBW buffer: Binding/Washing buffer; 10mM Tris pH 7.5, 1mM EDTA, 2M NaCl

The amplification products are then ready for sequencing as follows. A reaction mixture is prepared consisting of 2ul T7 Thermo Sequenase™ buffer (Amersham Life Sciences, Cleveland) (final: 26 mM Tris-HCl, pH 9.5, 6.5 mM MgCl<sub>2</sub>); 3ul of PCR product from above; 3ul (final: ~30ng/5pM) Fluoresceinated primer; 3ul dH<sub>2</sub>O; and 2ul diluted Thermo Sequenase™ enzyme (Amersham Life Sciences, Cleveland) (final 6.4U). The total volume of 13 ul is kept on ice.

The fluoresceinated primer used has the same sequence as the non-biotinylated primer used in the amplification reaction, above, but it is fluoresceinated so that it may be detected on an A.L.F. Automated DNA Sequencing Apparatus (Pharmacia, Inc.; Piscataway, N.J.)

The reagents are mixed well, and a 3 ul aliquot is added to each of the 4 termination reaction tubes containing 3ul of Termination Mix. The final volume of 6 ul is covered with 10 ul mineral oil before thermal cycling.

The d/ddA Termination mix contains 750uM dNTPs, 2.5uM ddATP.

The d/ddC Termination mix contains 750uM dNTPs, 2.5uM ddCTP.

The d/ddG Termination mix contains 750uM dNTPs, 2.5uM ddGTP.

The d/ddT Termination mix contains 750uM dNTPs, 2.5uM ddTTP.

The sequencing reactions are then thermal cycled in a Perkin Elmer 9600 as follows:

94°C 2min	x1 cycle
94°C 30 sec	
*50°C 10 sec	cycle 25 times
70°C 30 sec	

70°C 2 min                      x1 cycle

\* annealing temperature may vary within about 6°C depending on empirically determined optimization for the specific amplification primer.

5                      After the cycle sequencing reaction is complete, 6 ul of STOP buffer comprising dextran blue in formamide is added to the reaction mixture. 6 ul or half of the reaction products are loaded onto an A.L.F. Sequencer.

10                      Example 4

Single-Tube Sequencing of a BRCA1 exon may be achieved as follows.

15                      The primer pair is selected from table 1 and is specific for the designated exon of BRCA1. Asymmetric amplification of the sequencing template strand is obtained using an excess ratio (10-50 fold) of template strand primer compared to the non-template strand primer. The following reactants were combined in an Eppendorf tube at 4 degrees C in the following amounts:

20                      300 ng patient sample genomic DNA

Primer 1: 1 pmole

Primer 2: 20 pmole

1 X Thermo Sequenase™ buffer (Amersham Life Sciences, Cleveland) (final: 26 mM Tris-HCl, pH 9.5, 6.5 mM MgCl<sub>2</sub>)

25                      5% DMSO

0.2 mM dNTPs (i.e. 0.05 of each dATP, dCTP, dGTP, dTTP)

0.25 units Thermo Sequenase™ enzyme (Amersham Life Sciences, Cleveland)

Final reaction volume: 4 microlitres. This small volume allowed for the reaction to proceed close to completion, and particularly to consume available dNTPs during the amplification step. The reaction was overlaid with 10 microlitres of Chill Out Liquid Wax.

The reaction tube was placed in a Perkin-Elmer 9600 Thermo-Cycling apparatus and thermal cycled as follows:

94°C 2 min                      x1 cycle

94°C 30 sec

\*60°C 30 sec                      x39 cycles

72°C 2 min

65°C 7 min                      x1 cycle

\* annealing temperature may vary within about 6°C depending on empirically determined optimization for the specific primer pair.

After thermal cycling, the reaction vessel was cooled to 4 C for 10 min, whereupon the Chill Out Wax solidified and prevented any PCR carry over of product which could lead to contamination.

6 microliters of the following sequencing mix was then added:

1.2 microliters: Sequencing Primer (20 pmole)

1.0 microliters concentrated Thermo Sequenase™ buffer (Amersham Life Sciences, Cleveland) (final: 26 mM Tris-HCl, pH 9.5, 6.5 mM MgCl<sub>2</sub>)

1.0 microliters thermal stable polymerase (Thermo Sequenase) (3 units)

1.0 microliters 20% (v/v) DMSO

3.0 microliters 1:100 ratio of ddNTP and 4 dNTPs (final concentrations of 2.5 microM and 250 microM respectively).

The Sequencing primer selected was a fluoresceinated version of the subservient primer (i.e. the one originally added in lesser amount). The fluorescent label allows for detection of reaction products in an automated DNA sequencer, such as the Pharmacia A.L.F.

The ddNTP selected corresponds to the desired termination reaction, and is either ddATP, ddCTP, ddGTP or ddTTP.

The reaction was thermal cycled at the following temperatures in a Perkin-Elmer 9600 thermal cycling apparatus for 15 cycles

95° C 15 sec

50° C 5 sec

70° C 15 sec

After the thermal cycles, the reaction was cooled to 4  
C. It was mixed with 6 microliters stop solution (formamide  
and glycerol), and 1-3 (up to 10) microliters were loaded per  
lane of an A.L.F. automated sequencer.

#### Example 5

Multiplex amplification of fragments of exon 11 listed  
in Table 1 as fragments 11b, 11d, 11i and 11l, of BRCA1 was  
achieved as follows:

5 ul of patient sample genomic DNA (20 ng/ul) was  
combined with 2 ul 10X PCR Buffer, 0.6 ul 50mM Mg2+, 0.4 ul 10  
mM dNTP mix (containing each of the 4 dNTPs), 1 ul DMSO (100%),  
and 6 ul ddH2O. 1 ul of a mixture containing 50 ng/ul of each  
primer pair (indicated in Table 1), (i.e. 50 ng each primer),  
one of each pair being labeled on its 5' end with a fluorescein  
label, was added to the mix, totaling 4 ul (1 ul for each  
primer pair).

The mixture was prepared on ice. Addition of 1ul Taq  
Polymerase (1U/ul) (Roche Molecular Systems, Inc.), brought the  
total volume to 20 ul, and was followed by thermal cycling as  
follows:

Initial denaturation 94C 10 min

Cycle (20 - 35 times):

anneal 50C 40 sec

extend 72C 60 sec

denature 94C 30 sec

Final extension 72C 10 min then held at room temperature.

An equal volume of Stop Solution comprising formamide and a visible dye was added after the reaction was completed. 6 ul of this mixture was loaded into a single lane of an A.L.F. Sequencing Gel, and the reaction products were detected on an A.L.F. automated DNA sequencer (Pharmacia, Inc., Piscataway, N.J.).

#### EXAMPLE 6

The procedure of Example 2 is repeated, using a combination of primers identified by Sequence ID Nos. 3, 4, 65 and 66 to perform a multiplex amplification of exons 2 and 20.